Evidence for Linkage Position Determination in Cobalt Coordinated Pentasaccharides Using Ion Trap Mass Spectrometry

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A methodology to determine the linkage position of oligosaccharides is presented. In order to illustrate this technique, several oligosaccharides and disaccharides were ionized by electrospray and analyzed in a Paul trap mass spectrometer. Multiple stage tandem mass spectrometry experiments were used to determine linkage and structural information for the following four cobalt coordinated and singly charged ($[M+Co-H]^+$) pentasaccharides: Lacto-*N*-fucopentaose I, II, III, and V. In order to differentiate between linkage positions, multiple low energy collision induced experiments with mass selected C type ions have been carried out in an ion trap mass spectrometer. Because of the coordination with cobalt, which directs the dissociation pathways, these C type ions undergo specific fragmentation reactions upon low energy collision induced dissociation. These dissociation pathways are unambiguously dependent on their linkage position, thus allowing differentiation between 1→2, 1→3, 1→4, and 1→6 linkage positions throughout the oligomers. Studies on various linked disaccharides and *N*-acetyl-disaccharides, which are smaller constituents of the pentasaccharides, were used to verify and confirm the results obtained from the pentasaccharides. (J Am Soc Mass Spectrom 1998, 9, 1125–1134) © 1998 American Society for Mass Spectrometry

Carbohydrates and carbohydrate containing compounds are the most common biomolecules in nature. However, there are few analytical methods and techniques available for complete structural analysis (monosaccharide composition, sequence, and linkage information). The wealth of information on structural analysis of carbohydrates using mass spectrometry (ms) is staggering and too numerous to give credit to all within the scope of this manuscript [1–17]. However, many of these are landmark papers and much of the methodology cited in these references are implemented today. In fact, classical chemical reactions involving oxidation, reduction, and peracetylation, some of which were originally reported 20 years ago, are still commonly used in combination with mass spectrometry for determining linkage position [2, 9–17]. These methods are still quite viable and routinely used, however, many are cumbersome, require large amounts of material, demand long analysis time, and often do not provide 100% unambiguous information.

Carbohydrates show a great variety of structural types with different chemical and physical properties. So far there exists no single analytical technique or single analytical method which could be used for total structural analysis for this class of biomolecules without using an inordinately large amount of material. Recent research has shown that both linkage and anomericity can be determined using chemical shifts of structural reporter groups via NMR spectroscopy [18, 19]. Differences, however, for these shifts can be extremely small, some as little as 0.002 ppm and spectra are extremely complex [19]. The biggest disadvantages in using NMR are sample purity and concentration requirements. Analysis of an unknown oligosaccharide of moderate length (penta- to octamer) by two-dimensional (2D) techniques requires 1–3 μmol of pure compound and can take 12 to 20 h for data collection and processing. In some cases, as much as 10 mg was needed to acquire a COSY spectrum of a sialic acid polysaccharide [20]. Research presented herein shows that we can differentiate linkage position with ion trap mass spectrometry using as little as 20 pmol of the metal-complexed pentamer.

Because the role of carbohydrates in biological and pharmaceutical applications is growing, the development of new and fast analytical methodologies is a major topic of current analytical research. In the past few years mass spectrometry, among a few other methods, has become one of the most powerful and versatile techniques for structural analysis of carbohydrates. Mass spectrometry, and in particular tandem mass spectrometry (MS/MS), provide many advantages over traditional analytical methods [21], such as low sample consumption, high sensitivity as well as obtaining

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structural information by MS/MS or MS^n experiments. Two recent articles have shown the versatility of electrospray ionization-ion trap mass spectrometry (ESI-ITMS) as a tool for structural determination of oligosaccharides emphasizing high sensitivity [22] and the ability to characterize permethylated oligomers [23].

In this laboratory, studies have focused on the development of new methodologies for structural identification of carbohydrates based on metal ion coordination and tandem mass spectrometry [24–33]. In earlier investigations we presented several methodologies to distinguish linkage position for disaccharides using lithium cationization [24–26]. Similar data were presented for branched oligomers using ESI with alkaline earth and transition metals [27, 28]. More recent MS/MS studies of diastereomeric metal *N*-glycoside complexes have focused on differentiation of stereochemical features of saccharides using either fast atom bombardment (FAB) or ESI ionization [29–32]. These data indicated that metal/ligand derivatization of hexoses could be used to unambiguously differentiate the four stereoisomers (Glc, Gal, Man, and Tal) [32].

Another approach, based on more recent advances in this laboratory, was the combination of ESI and ion trap technology with an emphasis on multiple stage experiments. Glish et al. [34] first reported results using this specific technique on the behavior of several lithium and sodium coordinated di-, tri-, and tetrasaccharides where they studied the dissociation pathways by MS^n and double resonance experiments.

Recently we have shown that when cobalt is used as the coordinating metal, structural and linkage information of *N*-acetyl containing oligosaccharides can be obtained [28]. In the present study, we have investigated a variety of disaccharides and pentasaccharides and our goal is to demonstrate the versatility of this methodology. Although other existing and reliable techniques are used for linkage determination, as cited above, metal ion derivatization followed by tandem mass spectrometry can also be successfully implemented on small amounts of material and data obtained in less than 30 min. We present data that demonstrate the feasibility of our approach.

Experimental

Instrumentation

All experiments were carried out on a Finnigan LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an ESI source. Fused silica lines between the syringe pump and electrospray probe were purchased from Polymicro (Phoenix, AZ) and the connection pieces (consisting of Peek and stainless steel with KEL-F fittings) were obtained from Upchurch Scientific (Oak Harbor, WA). Ionization was either performed under normal electrospray conditions (flow rate: 1 μL/min, 4.5 kV, sheath gas flow: 40 arb) or microspray conditions (flow rate: 0.35 μL/min, 2.8–3.5 kV, no sheath gas). All spectra were acquired at a capillary temperature of 200°C and all ion guide voltages were tuned to maximize the abundance of the cobalt coordinated complex ($[M+Co-H]^+$) or for negative mode the deprotonated ion species ($[M-H]^-$). In order to generate a spectrum, typically 30–40 single scans were averaged. The pressure inside the vacuum chamber was 1.8×10^{-5} torr or less (uncorrected ion gauge reading) during all the experiments. A collision energy between 0.8 and 1.5 V_{p-p} was used for each CID stage (in each case the precursor beam attenuation was set between 0 and 10%) and a mass range between 2 and 4 Da was isolated prior to the CID experiment (the isolation width was increased with the number of multiple stage experiments in order keep as many ions as possible in the trap). Throughout this study the terminology for tandem mass spectrometry and low energy CID is used as follows: MS^2 means one isolation step followed by a CID experiment followed by a full scan, MS^3 stands for isolation → CID → isolation → CID → full scan, etc. During these studies we extended the conventional Finnigan electrospray source to a microspray [35] source which was operated at lower ESI high voltage (2.8–3.5 kV) with no sheath gas applied. For both source types the ionization conditions are approximately the same. Mass spectra looked identical over a range of ionization conditions (ESI high voltage and capillary voltage). Generally, spectra acquired under microspray conditions resulted in a better signal to noise ratio than spectra acquired under normal electrospray conditions.

Sample Preparation

The Lacto-*N*-fucopentaoses (LNFP) I, II, III, and V were purchased from Oxford Glycosystems (Abingon, UK) and $CoCl_2$ was obtained form Fisher Scientific (Fair Lawn, NJ). The disaccharides (O-linked pyranosyl hexoses) and all solvents (HPLC grade) obtained from Sigma (St. Louis, MO). *N*-Acetyl substituted disaccharides were purchased from Biocarb (Lund, Sweden). All chemicals were used without further purification and solutions were prepared in 1:1 acetonitrile:water (v/v). NMR spectra for the pentasaccharides were provided by the chemical manufacturer for proof of the isomeric purity (>98%).

The concentration of the carbohydrate and metal was 10 and 100 pmol/μL, respectively. Carbohydrate and metal salt solutions were mixed just prior to the experiments to prevent any degradation.

Results and Discussion

Four isomeric pentasaccharides coordinated to cobalt were analyzed by ESI and low energy CID mass spectrometry. These pentasaccharides, LNFP I, II, III, and V (Scheme **1**), each form prominent singly charged ions, $[M+CoCl]^+$ and $[M+Co-H]^+$ during ESI. Tandem mass spectrometry experiments, MS^n, of these precur-

Galβ1-3GlcNAcβ1-3Galβ1-4Glc LNFP I
2
|
Fucα1

Galβ1-3GlcNAcβ1-3Galβ1-4Glc LNFP II
4
|
Fucα1

Galβ1-4GlcNAcβ1-3Galβ1-4Glc LNFP III
3
|
Fucα1

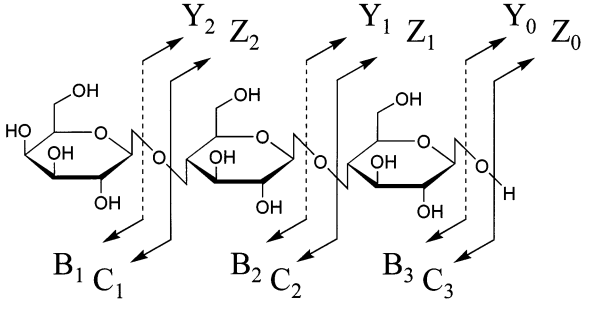
Galβ1-3GlcNAcβ1-3Galβ1-4Glc LNFP V
3
|
Fucα1

Scheme 1. Lacto-N-fucopentaose I, II, III and V.

sors and the corresponding C type ions [36] (Scheme **2** for nomenclature), clearly showed differences among the spectra which allowed the determination of the linkage throughout the pentasaccharides. Similar MS^2 studies on disaccharides, which make up the smaller portions of these pentasaccharides, assisted in determining the patterns of dissociation which then allowed extrapolation to larger oligomers [37].

All observed neutral losses from the different C type ions are summarized in Table 1 for each dissaccharide and in Table 2 for all four pentasaccharides. Table 1 includes the linkage, monosaccharide composition, and the observed neutral losses which are used to determine the linkage position. Table 2 includes the MS^n data (with mass-to-charge ratio of the C type ion in parentheses), the observed neutral losses used for linkage determination and the assigned linkage. In all cases, a cutoff of 3% relative abundance was used as a criteria for establishing presence or absence of ions of interest. We have found that this criteria is reproducible from run to run, sample to sample.

The results discussed below were obtained on 10 pmol/μL sample concentrations with a total consumption of no more than 20 pmol. The low consumption is a particular advantage of this methodology.



Scheme 2. Domon-Costello nomenclature for carbohydrate ions.

Table 1. MS^2 results obtained from CID experiments of $[M+Co-H]^+$ for the indicated disaccharides with specific neutral losses depending on their linkage position

Compound	Observed neutral losses for linkage determination			
Glc β 1–2 Glc	$-C_2H_4O_2$		$-C_4H_8O_4$	
Gal α 1–3 Gal		$-C_3H_6O_3$	$-C_4H_8O_4$	
Gal α 1–4 Gal	$-C_2H_4O_2$	$-C_3H_6O_3$	$-C_4H_8O_4$	
Gal α 1–6 Glc	$-C_2H_4O_2$			
Gal β 1–3 GalNAc				−162
Gal β 1–4 GlcNAc				−161

LNFP III

The MS^2 spectrum of the deprotonated precursor ($[M+Co-H]^+$) of LNFP III, Figure 1, shows three neutral losses: $C_2H_4O_2$, $C_3H_6O_3$, and $C_4H_8O_4$ at m/z 851, 821, and 791, respectively. Concurrent MS^2 experiments of various linked hexose disaccharides and *N*-acetyl-disaccharides were carried out in order to determine which neutral losses were associated with which linkage position and these results are summarized in Table 1. According to Table 1, the three neutral losses described above for LNFP III should indicate a 1→4 linkage, which in fact describes the reducing end linkage for that compound. We know from our previous studies that dissociation of these metal coordinated oligomers begins from the reducing end with these specific cross ring cleavages and then proceeds to the nonreducing end in a step wise fashion [24–26].

The ion at m/z 749 in Figure 1 is a glycosidic C ion and it is this ion which is selected for MS^3 studies; i.e., 911→749→. The MS^3 experiment is shown in Figure 2. Two prominent ions at m/z 659 and 629 are observed which represent neutral losses of $C_3H_6O_3$ and $C_4H_8O_4$, respectively. Reference to Table 1 suggests that these losses are indicative of a 1→3 linkage, which again agrees with the penultimate linkage from the reducing ring of the cobalt coordinated LNFP III complex. As in the MS^2 experiment, the next glycosidic C type ion appears at m/z 587 (two additional examples of this ability to distinguish the 1→3 linkage position are provided in the supplementary material for LNFP I and II).

The MS^4 experiment (911→749→587→) shows an ion of 100% abundance at m/z 411 corresponding to the loss of a fucose moiety (Figure 3). At this point in the interpretation we know that the trisaccharide at m/z 587 loses fucose and that the remaining disaccharide contains one *N*-acetyl hexosamine. Unfortunately, we cannot at this stage determine what linkage position the fucose occupies. This, however, is determined from complementary MS^2 experiments of the deprotonated pentamer which is discussed further on [MS^2 experiments on $(M-H)^-$].

The MS^5 experiment (911→749→587→441→) is shown in Figure 4 and a proposed mechanism for the formation for m/z 280 presented in Scheme **3** (prece-

Table 2. MS^n results obtained from CID experiments of the cobalt coordinated LNFPs, listing the specific neutral losses, MS^n experiment with the precursor ion in parentheses, corresponding mass-to-charge ratio, and the resulting linkage position

	MS^n	$-C_2H_4O_2$	$-C_3H_6O_3$	$-C_4H_8O_4$	−162	−161	Linkage
LNFP III	MS^2 (911)	851	821	791			1 → 4
	MS^3 (749)		659	629			1 → 3
	MS^4 (587)						
	MS^5 (441)					280	1 → 4
LNFP V	MS^2 (911)						
	MS^3 (765)	705	675	645			1 → 4
	MS^4 (603)		513	483			1 → 3
	MS^5 (441)				279		1 → 3
LNFP I	MS^2 (911)	851	821	791			1 → 4
	MS^3 (749)		659	629			1 → 3
	MS^4 (587)						
	MS^5 (441)				279		1 → 3
LNFP II	MS^2 (911)	851	821	791			1 → 4
	MS^3 (749)		659	629			1 → 3
	MS^4 (587)						
	MS^5 (441)				279		1 → 3

dence for alkoxide driven reactions have been previously reported by this group [25, 27, 28]). In the case of LNFP III, the ion at m/z 441 decomposes to an ion at m/z 280 representing a neutral loss of 161 Da. Concurrent MS^2 experiments on 1→3 Gal-GalNAc and 1→4 linked Gal–GlcNAc dissaccharides suggest two different pathways for the respective losses of 161 and 162 Da as indicated in Table 1. MS^2 of the perdeuterated (exchangeable protons only) or ^{18}O labeled (anomeric oxygen) Gal–GlcNAc suggests a possible dissociation mechanism for the formation of the m/z 280 ion as shown in Scheme **3**. Deprotonation at C−3 position of the reducing ring of the 1→4 linked dimer and subsequent electron migration from the alkoxide generates the loss of two neutral species and regenerates the alkoxide as shown. We have observed previously a similar C−3 hydroxyl deprotonation site for 1→4 linked oligomers and both labeling experiments support this mechanism [38]. This is different from the mechanism proposed for the 1→3 Gal–GalNAc shown in Scheme **4**. In this latter case the neutral hexose epoxide is lost as evidenced by the labeling experiments.

Given the MS^2 through MS^5 studies, in conjunction with the disaccharide experiments, one would thus predict the linkages to be 1→3, 1→3, 1→4 from the reducing to the nonreducing end. Fucose is known to be associated with the trimeric nonreducing portion as evidenced by the reduction in mass, but at this point its linkage is still not known (see further discussion below).

LNFP V

The MS^2 spectrum of the precursor ion ($[M+Co-H]^+$) of LNFP V shows a loss of 146 Da, which represents a loss of dehydro fucose (Figure 5). From earlier results and reports from other research groups we know that this dissociation pathway is very typical for a loss of a 1→3 linked fucose at the reducing end [27, 39]. The determination of the linkage position of a fucose sub-

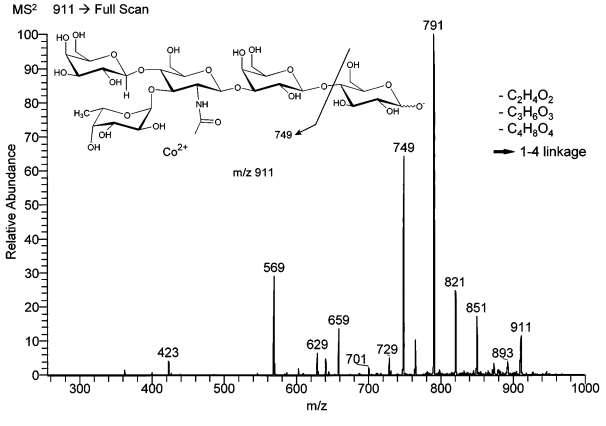


Figure 1. MS^2 (911→) spectrum of cobalt coordinated LNFP III $[M+Co-H]^+$.

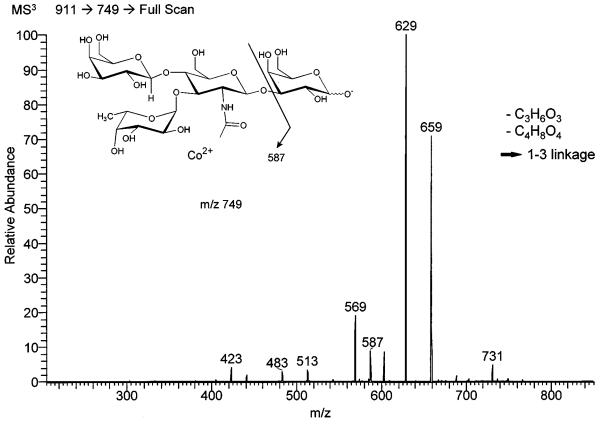


Figure 2. MS^3 (911→749→) spectrum of cobalt coordinated LNFP III $[M+Co-H]^+$.

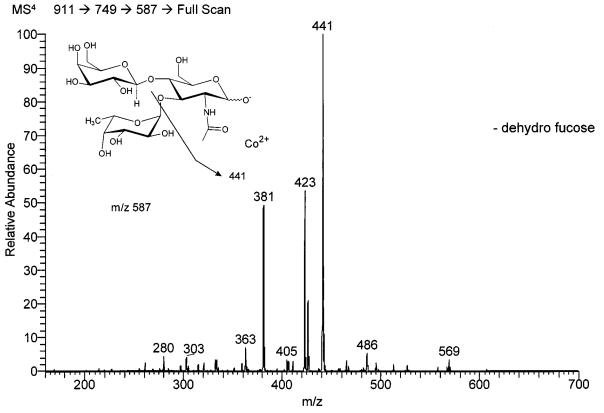


Figure 3. MS^4 (911→749→587→) spectrum of cobalt coordinated LNFP III $[M+Co-H]^+$.

stituent is often difficult and requires additional information from specific reaction pathways as shown here or from additional biological information. It is noteworthy to mention the ions at m/z 705, 675, 645 which represent $C_2H_4O_2$, $C_3H_6O_3$, and $C_4H_8O_4$ losses from m/z 765, respectively, and an ion at m/z 603 which represents the C_3 ion. We have observed that the loss of fucose occurs at low collision energy and often the main fragment ion (a glycosidic cleavage at m/z 765) gains enough internal energy during collisional activation to continue dissociating. Therefore, we can observe cross ring cleavages at m/z 705, 675, and 645 and the next glycosidic ion at m/z 603. These observed losses from the glycosidic ion at m/z 765 suggests a 1→4 linkage which in fact represents the penultimate linkage from the reducing end. However, in order to proceed with our proposed methodology we subsequently performed an MS^3 experiment (911→765→) on the C_4 ion at m/z 765.

The MS^3 experiments (Figure 6) shows indeed the same neutral losses as described above in the MS^2 experiment; i.e., neutral losses of $C_2H_4O_2$, $C_3H_6O_3$, and $C_4H_8O_4$ at m/z 705, 675, and 645, respectively, and the next glycosidic ion at m/z 603. By comparing these

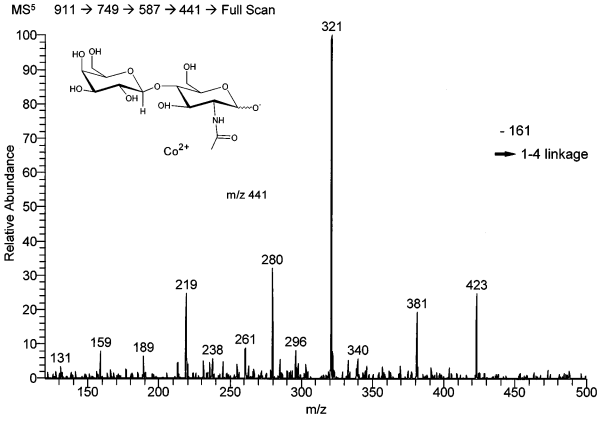
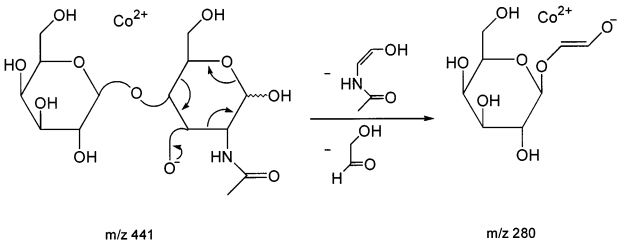


Figure 4. MS^5 (911→749→587→441→) spectrum of cobalt coordinated LNFP III $[M+Co-H]^+$.

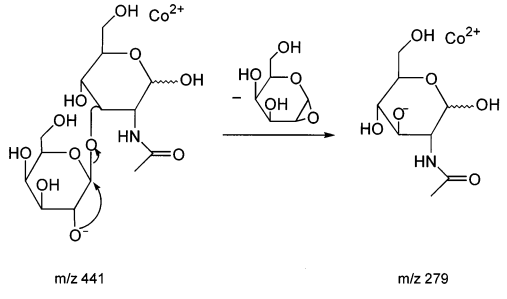


Scheme 3. Dissociation mechanism for 1→4 linked Gal-GalNAc, based on ^{18}O (anomeric position) and ^2H (exchangeable protons) labeling experiments.

observed neutral losses with Table 1 we can confirm a 1→4 linkage position between the lost hexose and the remaining trimer, which at this point of the analysis suggests a 1→3 and 1→4 linkage position form the reducing to the nonreducing end. This sequence again agrees with the linkage appearing in LNFP V.

During the MS^4 experiment (Figure 7) the C type ion at m/z 603 undergoes neutral losses of $C_3H_6O_3$ and $C_4H_8O_4$ to give m/z 513 and m/z 483 indicating a 1→3 linkage position according to Table 1. The glycosidic cleavage from the precursor ion at m/z 603 results in the next C type ion at m/z 441.

In the final MS^5 experiment (Figure 8) we observe a neutral loss of 162 giving rise to a fragment ion at m/z 279 indicating a 1→3 linkage position between Gal and GlcNAc. The dissociation pathways in this case is somewhat different from LNFP III; deprotonation occurs on the C−3 position and leads to a loss of the dehydro hexose linked at the C−3 carbon of the GalNAc moiety. Once again, this mechanism is supported by labeling experiments [38] (^{18}O at the anomeric position and ^2H for the exchangeable protons) on the corresponding disaccharides, and is shown in Scheme **4**. The resulting linkage sequence in LNFP V therefore must be 1→3, 1→4, 1→3, and 1→3 from the reducing to the nonreducing end which accurately represents the linkages in LNFP V. With the complementary information about the loss of fucose on the reducing end, we were able to obtain information on all linkages by performing MS^n experiments on the cobalt coordinated oligosaccharide complex ($[M+Co-H]^+$).



Scheme 4. Dissociation mechanism for 1→3 linked Gal-GalNAc, based on ^{18}O (anomeric position) and ^2H (exchangeable protons) labeling experiments.

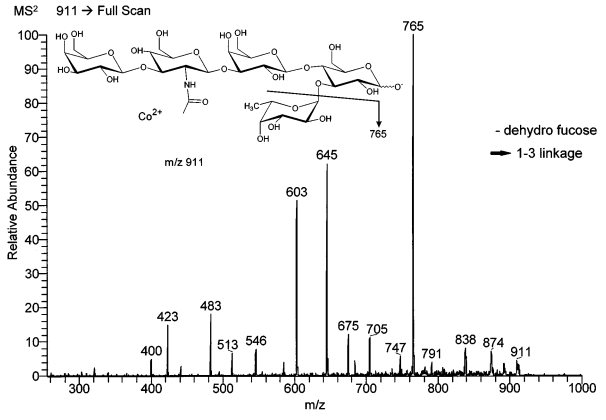


Figure 5. MS^2 (911→) spectrum of cobalt coordinated LNFP V $[M+Co-H]^+$.

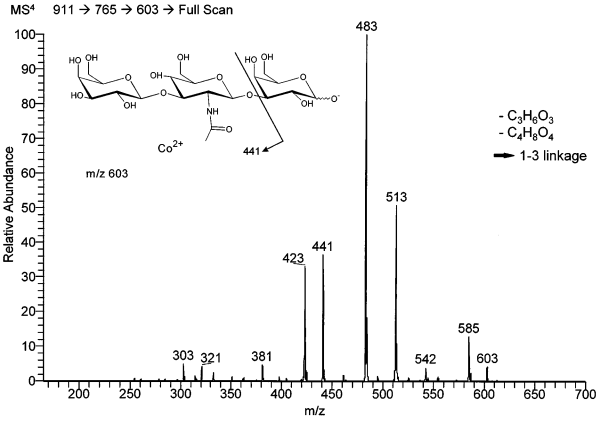


Figure 7. MS^4 (911→765→603→) spectrum of cobalt coordinated LNFP V $[M+Co-H]^+$.

LNFP I

The MS^2 experiment (911→) of $[M+Co-H]^+$ of LNFP I shows three neutral losses, $C_2H_4O_2$, $C_3H_6O_3$, and $C_4H_8O_4$ at m/z 851 (48%), 821 (17%), and 791 (100%), respectively as outlined in Table 2 (all spectra provided in supplementary material). Referring to Table 1, the three observed neutral losses described above are indicative for a 1→4 linkage, which agrees with the reducing end linkage.

The ion at m/z 749 (38%) represents the next glycosidic C type ion and is used for the MS^3 experiment (911→749→). The MS^3 spectrum features the intense ions at m/z 659 (60%) and 629 (100%) which represent neutral losses of $C_3H_6O_3$ and $C_4H_8O_4$, respectively. Reference to Table 1 suggests that these losses give evidence of a 1→3 linkage, which in fact describes the penultimate linkage from the reducing ring of the cobalt coordinated LNFP I complex. Ions at m/z 603 (48%), 587 (45%), and 569 (46%) represent a loss of fucose, the next glycosidic ion and a loss of water, respectively. These ions do not provide any further linkage information.

The MS^4 experiment (911→749→587→) features an ion of 100% abundance at m/z 411 corresponding to the loss of a fucose moiety, but the linkage position of the fucose cannot be determined at this point. A complementary MS^3 experiment of the deprotonated pentamer which is discussed further on (MS^2 experiments on $(M-H)^-$) makes it possible to assign the fucose linkage position.

In the MS^5 experiment we observe a neutral loss of 162 at m/z 279 (19%) which can be associated with a 1→3 linkage between the hexose and the HexNAc unit at the nonreducing end. Reaction mechanisms are identical as described for LNFP V (Scheme 4). With this information obtained by the MS^2 through MS^5 experiments a linkage sequence of 1→4, 1→3, 1→4 is predicted from the reducing to the nonreducing end, which agrees with the known linkage sequence of LNFP I. The fucose moiety has to be bonded to the nonreducing ring, but its location and linkage position cannot be determined at this point. However, this missing information can be obtained by MS^2 (852→) and MS^3 (852→325→) experiments on the deprotonated pentamer, as discussed further on [MS^2 experiments of $(M-H)^-$].

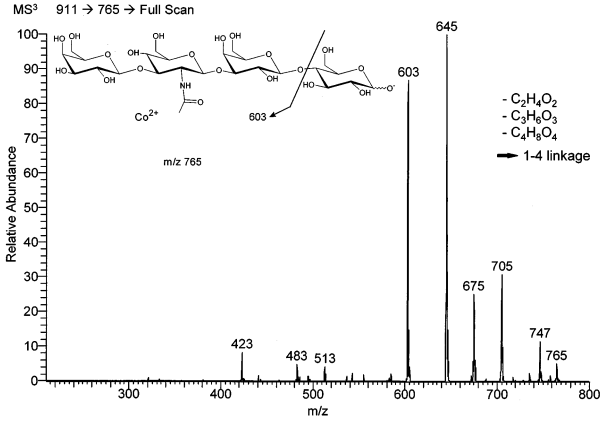


Figure 6. MS^3 (911→765) spectrum of cobalt coordinated LNFP V $[M+Co-H]^+$.

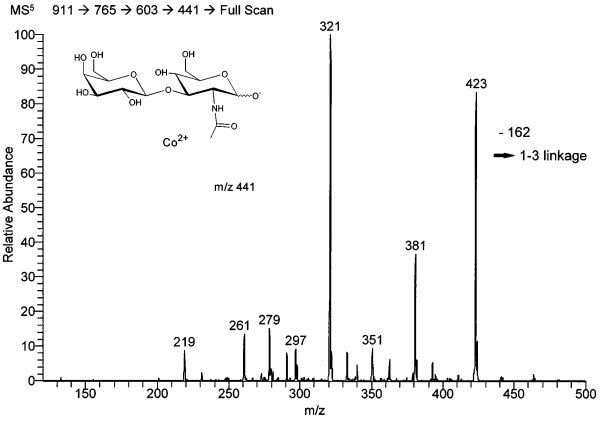


Figure 8. MS^5 (911→765→603→441→) spectrum of cobalt coordinated LNFP V $[M+Co-H]^+$.

Table 3. Observed neutral losses from the precursor $(M-H)^-$ of each LNFP

LNFP III	MS^2 (852)	dehydro Glc (*m/z* 690)	dehydro Gal (*m/z* 528)	Fuc (*m/z* 364)
LNFP V	MS^2 (852)	Fuc (*m/z* 688)	$C_6H_8O_4$ (*m/z* 544)	dehydro Gal (*m/z* 382)
LNFP I	MS^2 (852)	dehydro Glc (*m/z* 690)	dehydro Gal (*m/z* 528)	dehydro GalNAc (*m/z* 325)
LNFP II	MS^2 (852)	dehydro Glc (*m/z* 690)	dehydro Gal (*m/z* 528)	Gal (*m/z* 348)

LNFP II

The MS^2 spectrum of the deprotonated precursor ion species ($[M+Co-H]^+$) of LNFP II features three neutral losses, $C_2H_4O_2$, $C_3H_6O_3$, and $C_4H_8O_4$ at *m/z* 851 (30%), 821 (28%), and 791 (100%), respectively, as observed in Table 2 (all spectra provided in supplementary material). According to the neutral loss table for the disaccharides (Table 1), the three neutral losses described above for LNFP I can be assigned to a 1→4 linkage, which again confirms the reducing end linkage for that compound. The next glycosidic ion appears at *m/z* 749 (65%) and is used for the MS^3 studies (911→749→).

In the MS^3 spectrum two prominent ions at *m/z* 659 (70%) and 629 (100%) are observed that represent neutral losses of $C_3H_6O_3$ and $C_4H_8O_4$, respectively, as listed in Table 2. This suggests that these losses are an indication of a 1→3 linkage, which describes the penultimate linkage from the reducing ring of the cobalt coordinated LNFP II complex.

The MS^4 experiment (911→749→587→) shows an ion at *m/z* 411 (100% abundance) corresponding to the loss of a fucose moiety. At this stage we know that the remaining trisaccharide moiety at *m/z* 587 loses fucose and that the ensuing disaccharide contains one HexNAc based on differences in masses.

In the final MS^5 experiment (911→749→587→441→) we see a neutral loss of 162 at *m/z* 279 (18%) which can be associated with a 1→3 linkage in the remaining disaccharide which must be a Hex–HexNAc portion given by the appearance of *m/z* 441. Again this reaction mechanism is identical to that described for LNFP V (Scheme **4**). With the information obtained from the MS^2 through MS^5 experiments, a linkage sequence of 1→4, 1→3, 1→4 is predicted from the reducing to the nonreducing end. This agrees with the linkage sequence for LNFP II. The fucose moiety must be linked to the nonreducing ring, but its location and linkage position cannot be determined at this point (compare further discussion below).

MS^2 Experiments of $(M-H)^-$

Multiple stage tandem mass spectrometry on cobalt coordinated oligosaccharides can be used to obtain linkage information as we have shown in this study. However, supplementary MS^2 experiments on the singly deprotonated species, $(M-H)^-$, are required. We have noticed that in many cases these experiments provide very useful, sometimes even necessary additional structural information that can be used to verify or confirm results obtained with the cobalt coordinated species. The experiments can be performed with the same metal/carbohydrate mixture solution by switching from positive to negative electrospray mode and therefore sample preparation is minimized and consumption is minimal. The resulting spectra show the sequence of the oligosaccharides as sequential losses of dehydro hexoses (162 Da) or dehydro HexNAc units (203 Da). In addition, we have observed that the MS^2 experiments of the deprotonated species, $(M-H)^-$, allows the determination of 1→3 linkages on branch points. For example, in linear 1→3 linkage oligomers, losses occur as dehydro hexoses (162 Da); in branched systems these species are lost as hexose units (180 Da).

MS^2 experiments of the $(M-H)^-$ ions for all four pentamers are discussed below in the same order as they are above; LNFP III, V, I, and II. The neutral losses from the precursor ion at *m/z* 852, $(M-H)^-$, are also listed separately for each pentasaccharide in Table 3.

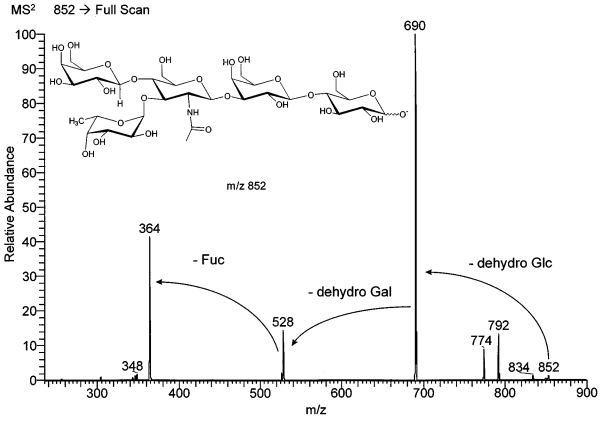


Figure 9. MS^2 (852→) spectrum of deprotonated LNFP III $(M-H)^-$.

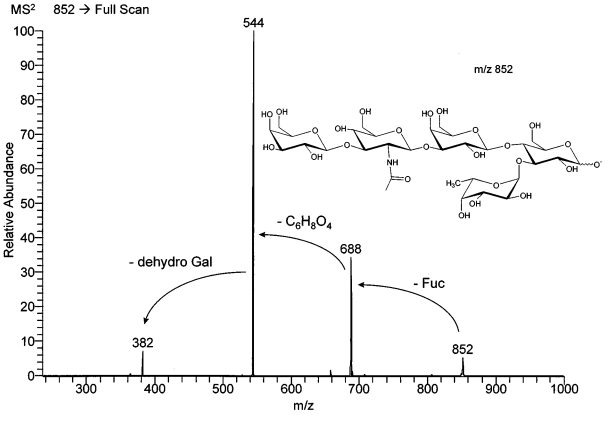


Figure 10. MS^2 (852→) spectrum of deprotonated LNFP V $(M-H)^-$.

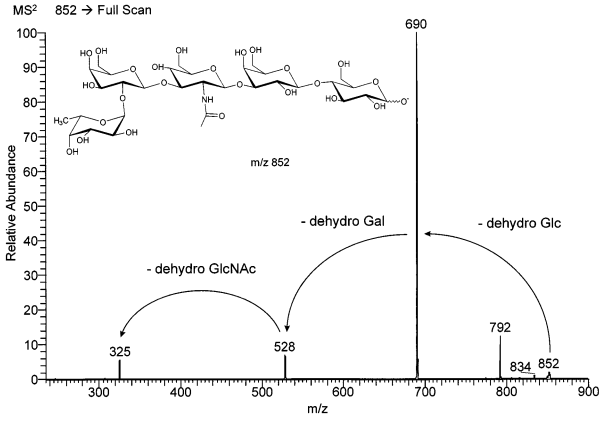


Figure 11. MS^2 (852→) spectrum of deprotonated LNFP I $(M-H)^-$.

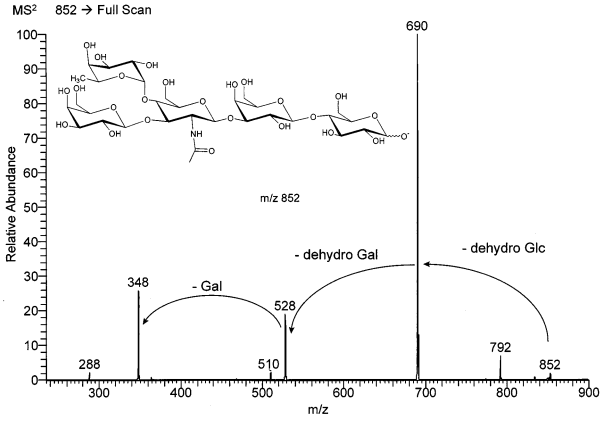


Figure 13. MS^2 (852→) spectrum of deprotonated LNFP II $(M-H)^-$.

LNFP III: The MS^2 spectrum of $(M-H)^-$ of LFNP III (Figure 9) shows three prominent ions at m/z 690, 528, and 364 which represent neutral losses of dehydro Glc, dehydro Gal, and fucose, respectively. The ions at m/z 792 and 774 represent losses of $C_2H_4O_2$ and $(C_2H_4O_2+H_2O)$ from the precursor ion. At this point in the interpretation we can also determine what linkage position the fucose occupies on the nonreducing end. The ion at m/z 364 represents a loss of fucose (and not dehydrofucose) from m/z 528, therefore the linkage must be 1→3, as described above.

LNFP V: The MS^2 experiment (Figure 10) of the precursor ion $(M-H)^-$ of LNFP V shows three major peaks at m/z 688, 544, and 382, which represent sequential losses of fucose, $C_6H_8O_4$, and dehydro Gal, respectively. In this case we can assign a 1→3 linkage between the fucose and the remaining tetramer because loss of fucose at m/z 688 is the first dissociation ion observed [25, 39]. This is a confirmation of the results obtained from the cobalt coordinated species. The two other fragments at m/z 544 (loss of fucose+$C_6H_8O_4$ from the precursor) and m/z 382 (loss of fucose+$C_6H_8O_4$+dehydro Gal from the precursor) give evidence of the sequence, but no further structural information.

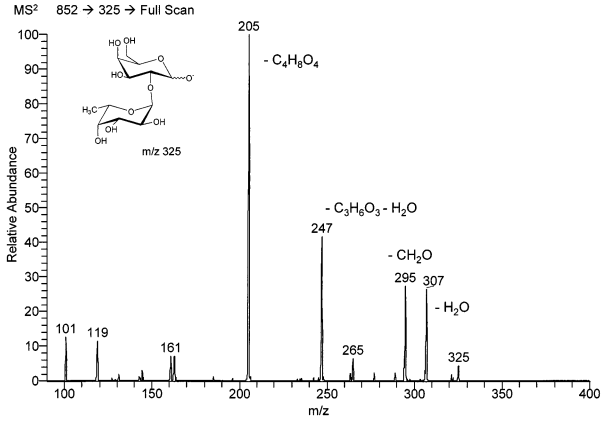


Figure 12. MS^3 (852→325→) spectrum of deprotonated LNFP I $(M-H)^-$.

LNFP I: In the MS^2 spectrum of $(M-H)^-$ of LNFP I we observe sequential losses of dehydro Glc, dehydro Gal, and dehydro GalNAc at m/z 690, 528, and 325, respectively (Figure 11). An ion at m/z 792 represents a loss of $C_2H_4O_2$ from the precursor ion, but cannot be used to gain any structural information. Given the results from the cobalt coordinated complex and the MS^2 spectrum of the deprotonated species, $(M-H)^-$ one would predict a linkage sequence of 1→4, 1→3, and 1→3 from the reducing to the nonreducing end with a fucose linked to the monosaccharide at the nonreducing end. The association of fucose is evidenced by reduction of mass in the MS^2 experiment (852→). In order to obtain the fucose linkage, an MS^3 spectrum of LNFP I (852→325→) was acquired. The MS^3 spectrum (Figure 12) shows a loss of water (m/z 307), a loss formaldehyde (m/z 295), loss of $C_3H_6O_3+H_2O$ (m/z 247), and a major loss of $C_4H_8O_4$ (m/z 205). From previous results we know that a major loss of $C_4H_8O_4$ is evidence of a 1→2 linkage [25].

LNFP II: The MS^2 experiment (Figure 13) of the precursor ion at m/z 852 features three major peaks at m/z 690, 528, and 348 which represent sequential loss of dehydro Glc, dehydro Gal, and Gal. The ion at m/z 792 is a loss of $C_2H_4O_2$ from the precursor ion, which is also present in the MS^2 spectrum of LNFP I and V because they have the same monosaccharide ring at the reducing portion. At this point we know the linkage positions of 3 of the 4 linkages. The sequence must be 1→4, 1→3, and 1→3 from the reducing to the nonreducing end. Given by the reduction in mass and the loss of a Gal unit, which gives the evidence of a branch point, the fucose moiety must be on the GlcNAc portion. Unfortunately, the remaining linkage between the fucose and the remaining disaccharide Gal–GlcNAc moiety cannot be determined by any of the presented methods; it can be either 1→4 or 1→6. This is the only

linkage we were not able to assign by our technique. However, in many cases one might have some additional biological information which makes it possible to assign the linkage.

An important observation during these sequencing experiments is noteworthy; by comparing the MS^2 spectra (852→) of LNFP I, II, III, and V we see very good agreement in terms of abundances of the ions at m/z 792, 690, and 528 where the structure of each of these compounds is identical and therefore the observed neutral losses should be identical. In fact, we see that the abundance of these three ions are almost equal. Given this observation we are very confident that all these dissociation reactions occur in the same time frame and at very narrow dissociation energy levels and therefore should provide reliable and reproducible results.

Extended MS^2 Experiments

Thus far we have shown that addition of an appropriate metal (in this case, cobalt) to an oligosaccharide solution provides one with the ability to determine linkage position using MS^n experiments. We were able to apply this method to pentasaccharides and smaller constituents of the pentamers. We propose that cobalt can also be used for larger carbohydrates and one concurrent topic of our research involves studies in this direction. This methodology works well for all compounds we have analyzed so far. However, we have encountered minor difficulties; one is the mass range limitation for tandem mass spectrometry on our instrument (the LCQ has currently an upper mass limit of 2000 Da) and the second problem is the MS/MS efficiency. Each tandem stage will give several product ion species (e.g., ions formed by cross ring cleavages and/or glycosidic cleavages) and the MS/MS efficiency can vary over a wide range. This is crucial for the intensity of the C type ions because these are essential for the determination of the linkage. With each additional stage of MS^n, the abundance of ions decreases until there are very few ions left in the trap for further experiments. We have found that this methodology becomes limited at MS^5 or MS^6. Consequently, we have tried to find a way of reducing the number of MS^n steps which must be carried out to obtain the same linkage information. In order to circumvent these limitations we have investigated ways to generate each C type ion throughout the oligosaccharide chain in the MS^2 experiment. By increasing the collision energy in the MS^2 experiments (for the $[M+Co-H]^+$ ion species at m/z 911) we were eventually able to generate each of the C type ions along the carbohydrate sequence and thus access them for an MS^3 experiment [we know which ions are C type from the MS^2 experiments of the $(M-H)^-$ ions]. The MS^3 experiments on each of these C type ions along the carbohydrate chain provided the same structural information and hence the linkage position. In order to illustrate this technique two spectra of LNFP III are compared (Figure 4 for the MS^5 experiment and Figure 14 for the MS^3 experiment). Both spectra result from the CID of the C_2 precursor ion. The first spectrum (Figure 4) was acquired as an MS^5 experiment (911→749→587→441→) and the second spectrum (Figure 14) as an MS^3 experiment (911→441→). Both spectra are very similar and according to Table 1 both spectra indicate a 1→4 linkage which is the linkage between Gal and GlcNAc for LNFP III at the nonreducing end. We have tested this method on a variety of compounds and found no difference in terms of linkage position determination between the MS^n and MS^3 spectra. If one intends to analyze larger oligosaccharides, the limitation will be of course the exclusion limit which defines the lowest mass-to-charge ratio stored inside the trap and is given by the instrumental parameter q_z. All experiments were performed at $q_z = 0.25$ which is the instruments default value. At this time we have not made any attempt to perform the MS^n experiments at lower q_z values. In order to analyze a larger oligosaccharide, such as a decamer, one would have to obtain the first linkage in an MS^2 experiment, then proceed with the described MS^3 experiments to gain the linkages as far as the exclusion limits allows. This would then be followed by selecting a C type ion close to the exclusion limit and proceeding with an MS^4 experiment in order to gain the remaining linkage information.

A significant advantage we encountered with this technique were fewer isolation and dissociation stages which shortened the acquisition time and therefore sample consumption was reduced. Time consuming MS^5 and MS^4 experiments were no longer required because all the linkage information could be obtained in an MS^2 and MS^3 experiment.

A notable benefit to this methodology is that typical sample consumption needed to obtain complete linkage information for a specific pentasaccharide was in the low pmol level. Thus far we have made no effort to reduce the sample consumption, but based on early preliminary experiments, we expect that the sample consumption can be further reduced by a factor of 100.

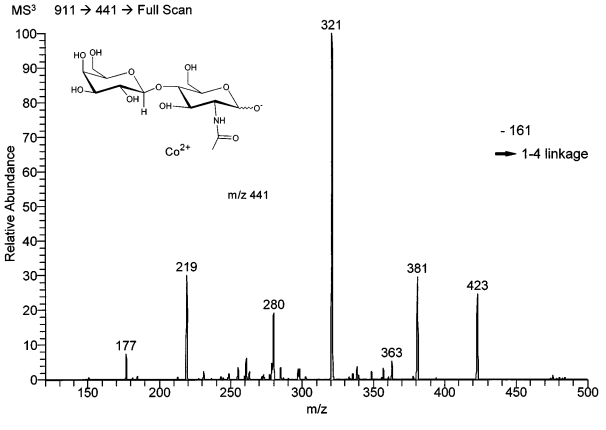


Figure 14. MS^3 (911→441→) spectrum of cobalt coordinated LNFP III $[M+Co-H]^+$.

Conclusions

This study has demonstrated a method to determine linkage position on four pentasaccharides (LNFP I, II, III, and V). Based on this methodology we were able to assign all linkages occurring in these pentamers except for one (site on LNFP II). Despite this minor deficiency, we propose that this technique can be used to obtain linkage positions on a great number and variety of different oligosaccharides.

Based on the investigation performed on the cobalt coordinated disaccharides a general table for fragmentation patterns of C type ions is presented which allows one to assign linkage in larger oligomers.

Former research in this laboratory has focused on the choice of the coordinating metal for linkage determination with the result that lithium could be used for straight chain oligomers and cobalt for systems possessing *N*-acetyl functionalities. Based on these results and ongoing investigations we assume that cobalt can be used for almost any size oligosaccharides to gain linkage information.

Furthermore, we were able to introduce experiments where the linkage information throughout the pentasaccharides was obtained in an MS^2 or an MS^3 experiment. With this technique we were able to shorten acquisition time, reduce the sample consumption and simplify the experimental procedures which makes the linkage position accessible in a straightforward manner.

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